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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group:	1651
Confirmation No.:	7977
Application No.:	10/634,292
Invention:	**Nano-Structured Polymers For Use As Implants**
Applicant:	Haberstroh et al.
Filed:	August 5, 2003
Attorney Docket:	3220-73239
Examiner:	Davis, Ruth A.

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. THOMAS J. WEBSTER

Mail Stop Non-fee Amendment
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22213-1450

I, Thomas J. Webster, declare as follows:

1. I am currently an Associate Professor for the Divisions of Engineering and Orthopedic Surgery at Brown University, and the director of the Nanomedicine Laboratory which designs, synthesizes, and evaluates nanomaterials for various implant applications. I received a Bachelors Degree in Science with a concentration in chemical engineering from the University of Pittsburgh (1995) and Masters (MS) and Doctorate of Philosophy degrees (Ph.D.) in biomedical engineering from Rensselaer Polytechnic Institute (M.S., 1997; Ph.D., 2000). My research has centered on the design, synthesis,

and study of nanophase materials for various implant applications. My lab group has generated 4 books, 33 book chapters, 85 invited presentations (including tutorials), 215 literature articles and/or conference proceedings, and 245 conference presentations.

2. I have read and understand the specification of the captioned application and the pending claims in the application. The pending claims of the captioned patent application are directed to a nano-structured synthetic implant comprising a polymeric material having a nano-sized surface feature having at least one dimension in the range from about 25 nm to less than 100 nm. I have knowledge of the results described below because the assays described below were conducted in my laboratory under my direction.

3. An exemplary *in vitro* experiment conducted using the claimed composition is shown in Exhibit B attached to this declaration. The results obtained from the experiment demonstrates the unique unexpected properties associated with materials having a nano-sized surface feature having at least one dimension (vertical structures) of less than 100 nm.

4. The results shown in Figs 1B and 1E of Exhibit B show a polymeric substrate having vertical dimension surface features of less than 100 nm, and more particularly less than 50 nm, possess a high affinity for fibronectin and collagen type IV, thus increasing the adhesion of endothelial cells. Conversely, a significant increase in surface roughness, as indicated by surface RMS value does not necessarily guarantee an increase in favorable protein adsorption or cellular adhesion. In particular, the data produced for substrate surfaces having 400 nm vertical (950 nm lateral) dimension surface features indicate that an increase in vertical surface feature dimension leads to a hydrophobic material with a low affinity for fibronectin and collagen type IV. In this manner, this study demonstrates that substrates comprising surface features having a vertical dimension of less than 100 nm can promote bioactive protein adsorption and endothelial cell adhesion more than substrates that comprise surface features having a vertical dimension of 100 nm or greater.

Appl. No 10/634,292
Declaration of Dr. Thomas J. Webster

All statements herein made of my own knowledge are true, and all statements herein made on information and belief are believed to be true; these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Dated: February 17, 2009

By:

Thomas J. Webster, Ph.D.

Nano-Structured Polymers For Use As Implants

Comparison of Lateral and Vertical Surface Features Influence on Vascular Cell Adhesion

METHODS

Substrate fabrication

Poly(lactic-co-glycolic acid) (PLGA) surfaces with submicron and nanoscale spherical surface features were created using a variation of a method previously described by Miller et al., J Biomed Mater Res A (2007);81A:678-84. 18mm diameter borosilicate glass coverslips (Fisher) were cleaned and degreased by soaking in acetone for 10 minutes, sonicating in acetone for 10 minutes, washing several times with dH_2O, soaking in 70% ethanol for 10 minutes, sonicating in 70% ethanol for 10 minutes, and washing several times with dH_2O. 300 µl of a 10 wt% solution of polystyrene beads of either 190 nm, 300 nm, 400 nm, or 950 nm diameter (Bangs Labs) were then dispersed onto the coverslips and allowed to evaporate in ambient air.

PS monolayers were secured to a 1 cm high x 18 mm diameter glass rod using a commercially available glass adhesive, which was in turn fixed to the bottom of a borosilicate Petri dish (60 mm diameter x 15 mm height, Fisher) using that same adhesive. Each dish contained a total of three such assemblies. These constructs were then used as templates to create inverse PDMS molds.

PDMS (Sylgard 184 silicone elastomer; Dow Corning) was mixed according to the manufacturer's directions (Curing Agent : Base, 1:10 Vol%), then exposed to vacuum (-25 in. Hg, 20 min.) to remove bubbles created during the mixing process. The resulting mix was then poured over the PS bead / glass constructs described above, filling the Petri dishes, then allowed to cure for 48 hr at room temperature. PDMS molds were then peeled away from their respective substrates, inverted, and placed in a slightly larger Petri dish. Additional PDMS was poured around the edge to fix the molds to the Petri dishes, preventing deformation due to organic solvents, specifically chloroform. All molds were then rinsed several times with chloroform to lift off any residual PS beads. This process created a series of 18 mm diameter x 1 cm deep PDMS wells with highly ordered, inverse casts of nanoscale beads on the bottom, which could then be used to create surfaces with spherical nanoscale surface features.

PLGA (50:50 wt:wt%; mol. wt. 12-16 x 103 g; Polysciences) was dissolved by sonicating at the ratio of 0.5 g PLGA : 8 mL chloroform for approximately 10 min, or until fully dissolved. The resultant solution was poured over the PDMS molds described above, then allowed to evaporate for 48 hours at room temperature. PLGA films were then peeled back from their molds, and cut into 12 mm diameter discs approximately 500 µm thick. Nano-smooth surfaces were created by dissolving PLGA in chloroform as described above, then depositing 300 µl of the solution on 12 mm diameter borosilicate glass coverslips (Fisher) that were cleaned and degreased using the procedure previously described. Once again, chloroform was allowed to evaporate for 48 hours. All surfaces

were then stored under vacuum (-20 in. Hg.) until use. Prior to cell culture experiments, surfaces were soaked in 70% ethanol for 24 hours, then ddH_2O for 24 hours. 12 mm diameter glass coverslips were used as a reference substrate for protein adsorption and cell adhesion experiments. Coverslips were cleaned and degreased as described above, then etched in 1 M NaOH for 1 hour. The coverslips were then washed in dH_2O and sterilized in an autoclave at 121 C for 1 hour.

Protein adsorption ELISA

Prior to each step described below, samples were washed twice with PBS. Samples were incubated for 1 hour with 5 µg bovine fibronectin (Sigma) in 500 µl PBS, 5 µg bovine collagen type IV (Abcam) in 500 µl PBS, or 500 µl MCDB-131 complete media in 24 well cell culture plates. Samples were then blocked by incubating in 1% BSA (Sigma) for 1 hour, then incubated with a 3:1000 solution of primary antibody to either fibronectin (Rabbit anti-bovine polyclonal; Chemicon) or collagen type IV (Rabbit anti-bovine polyclonal; Abcam) in 1% BSA.

After washing with 0.05% Tween 20 (Sigma) in PBS, samples were incubated with a horseradish peroxidase conjugated secondary antibody (Goat anti-rabbit IgG (H+L); Bio-Rad) then moved to a new 24 well cell culture plate. Samples were tested for HRP activity using an ABTS substrate kit (Vector Labs) according to the manufacturer's instructions. After 20 minutes, 200 µl of each sample solution were transferred to a 96 well cell culture plate. Light absorbance was measured using a spectrophotometer operating at a wavelength of 405 nm

Cell Culture

Rat aortic endothelial cells were purchased from VEC Technologies and used without further characterization. Cells were grown in MCDB-131 complete medium (VEC Technologies) in a sterile, humidified environment of 95% air, 5% CO_2, and passaged according to standard cell culture guidelines.

Prior to cell seeding, all samples were incubated in 1 mL of cell culture media for 1 hour in 12 well tissue culture plates (BD Falcon). Cells at a population number between 8 and 12, and approximately 90% confluence were washed twice with PBS, then typsinized and diluted into a counting volume of either MCDB-131 complete medium or DMEM for serum free tests. Cells were counted using a hemocytometer then seeded onto PLGA surfaces or borosilicate glass coverslips at a density of 3500 cells/cm^2. After a 4 hour cell adhesion period, samples were fixed for 10 minutes in 10% formalin buffer (Fisher), stained with Hoescht 33258 (Sigma) to visualize nuclei, and viewed using a Zeiss Axiovert 200M florescence microscope. Cell counts were recorded as the average of five random fields per sample. Representative florescence images of adhering cells were captured using a Hamamatsu 1394 ORCA-ERA camera.

Statistics

All experiments were run in triplicate, and repeated at least three separate times. Results were analyzed for statistical significance using Student's T-tests. Statistical significance was defined as a $p < 0.05$.

Results

SEM analysis (not shown) confirmed that polystyrene beads formed a close packed structure after evaporation of water-based suspension on borosilicate substrates. SEM further confirmed the diameter of each bead size used to be within the manufacturer's stated tolerances.

Protein Adsorption Assays (ELISA)

Raw data from protein adsorption studies was calibrated by dividing results for each substrate by relative surface area as determined using AFM. This was done since the controlling dimension for protein adsorption, a diameter of 1-2 nm, is significantly smaller than the submicron scale surface feature dimension present on the experimental PLGA surfaces. Thus, the total surface area available for protein adsorption increased on submicron structured as compared to smooth surfaces; proteins could adsorb to the peaks or valleys present on the experimental surfaces, or anywhere in between. This calibration was not performed on cell adhesion data, since the characteristic dimension of endothelial cells, approximately 10 μm, is far greater than the size of PLGA surface features present. Therefore, adhering cells must attach to several spherical surface features, meaning the total surface area available for cellular adhesion is identical for each experimental surface. Fibronectin and collagen type IV both behaved similarly in protein adsorption studies (Figure 1A & 1B), adsorbing in highest amounts on PLGA surfaces with roughly 5 nm and 19 nm vertical dimension (190 nm and 400 nm lateral dimension, respectively) surface features. Specifically, these surfaces demonstrated significantly ($p<0.05$) increased adsorption of fibronectin and collagen type IV as compared to smooth PLGA surfaces. Interestingly, adsorption of these two proteins to the most hydrophilic and hydrophobic experimental surfaces, smooth surfaces and those with roughly 400 nm vertical dimension (950 nm lateral dimension) surface features, respectively, was statistically equal.

Endothelial Cell Adhesion

4 hour cell adhesion experiments (Figure 1D & 1E) carried out in MCDB-131 complete media indicated that PLGA surfaces with roughly 5 nm and 19 nm vertical dimension surface features exhibit significantly ($p<0.01$) increased endothelial cell adhesion when compared to smooth PLGA surfaces. These surfaces also show significantly ($p<0.05$) increased endothelial cell adhesion when compared to their roughly 86 nm vertical dimension (300 nm lateral dimension) counterparts. 4 hour cellular adhesion experiments performed in DMEM without FBS showed no significant differences in cellular adhesion across all substrates, though adhesion was consistently

the highest on surfaces with 400 nm vertical dimension surface features. Adhesion on PLGA surfaces with 5 nm and 19 nm vertical dimension surface features was significantly ($p<0.05$) higher following experiments performed in MCDB-131 complete media as compared to serum free DMEM. Though not significant, ($0.05<p<0.1$) it is worth noting that endothelial cell adhesion on PLGA surface with 400 nm vertical dimension surface features was consistently higher when performed using serum free media as compared to complete media.

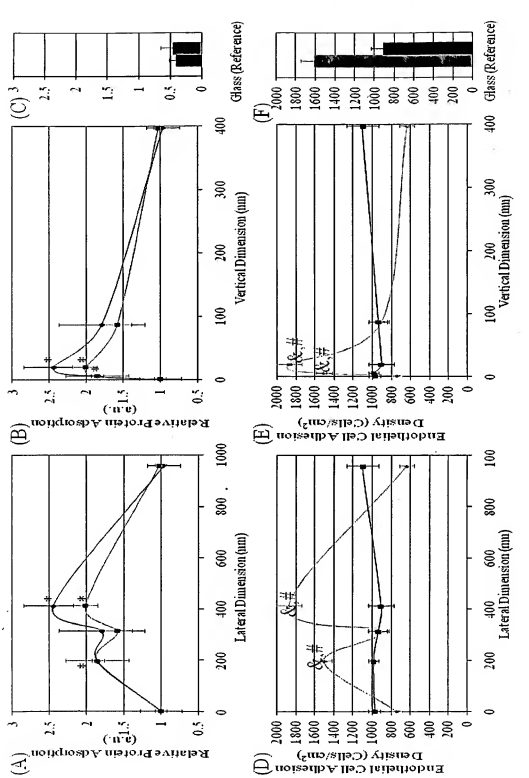
Comparing the results of cell adhesion experiments carried out in MCDB-131 complete media with the results of protein adhesion experiments, the two are strongly correlated. The correlation between each, defined as the covariance divided by the product of standard deviations, was found to be 0.944 between collagen type IV adsorption and cellular adhesion, and 0.965 between fibronectin adsorption and cellular adhesion. Additionally, when comparing all three data sets to vertical and lateral surface feature dimension, results appear to be dependent upon vertical, rather than lateral surface feature dimension. Specifically, plots of all three data sets against vertical surface feature dimension display a gamma distribution with an absolute maximum at a vertical dimension of approximately 19 nm, whereas plots against lateral surface feature dimension possess several local maxima without any clear x-y dependence.

Discussion

The results obtained from these experiments strongly suggest that it is the vertical dimension of submicron scale surface features, rather than the lateral dimension, that exerts the greatest influence on hydrophilicity and surface free energy. Additionally, a significant increase in surface roughness, as indicated by surface RMS value does not necessarily guarantee an increase in favorable protein adsorption or cellular adhesion. Such was the case here for surfaces with 400 nm vertical (950 nm lateral) dimension surface features. For those surfaces, an increase in vertical surface feature dimension led to a hydrophobic material with a low affinity for fibronectin and collagen type IV. Thus, for vascular tissue engineering applications in particular, surface features with a vertical dimension in excess of 100 nm may be undesirable, offering little or no practical advantage over conventional, smooth substrates, despite markedly higher complexity and costs of production. Instead, a surface with vertical dimension surface features of less than 50 nm should possess a high affinity for fibronectin and collagen type IV, thus increasing the adhesion of endothelial cells.

Figure legend:

Figure 1: **ELISA and Cell Culture Results** (A-C) Surface adsorption of collagen type IV (blue) and fibronectin (red), determined using ELISA. Results were calibrated for surface area and normalized to adsorption on smooth surfaces (a.u. denotes arbitrary units as a result of normalization), and compared to (A) lateral surface feature dimension as determined by AFM (B) vertical surface feature dimension as determined by AFM . Both proteins display almost equivalent behavior with regard to affinity for various surfaces. While adsorption of these two proteins bears no clear relationship to lateral surface feature dimension, it follows a gamma distribution with regard to vertical surface feature dimension, with a distinct maximum well below 100 nm. (D-F) Results of 4 hour endothelial cell adhesion test, performed using MCDB-131 complete media (green) or serum-free DMEM (black), and compared to (D) lateral surface feature dimension as determined by AFM (E) vertical surface feature dimension as determined by AFM. For experiments in MCDB-131 complete media, results are similar to those of protein adsorption experiments described above – while there is no clear relationship between cellular adhesion and lateral surface feature dimension, there is an apparent optimum centered around a vertical dimension well below 100 nm. Experiments performed in serum-free DMEM showed statistically equivalent results for all surfaces, implying that absorption of serum proteins is necessary to maximize cellular adhesion. Values are mean ± SEM, n=3. *: Protein adsorption significantly ($p<0.05$) increased as compared to smooth surfaces. &: Endothelial cell adhesion significantly ($p<0.01$) increased as compared to smooth surfaces and 950 nm lateral / 400 nm vertical dimension PLGA. #: Endothelial cell adhesion significantly ($p<0.05$) as compared to 300 nm lateral / 86 nm vertical dimension PLGA.


(A)
Relative Protein Adsorption (a.u.)
Lateral Dimension (nm)
(B)
Relative Protein Adsorption (a.u.)
Vertical Dimension (nm)
(C)
Glass (Reference)
(D)
Endothelial Cell Adhesion Density (Cells/cm²)
Lateral Dimension (nm)
(E)
Endothelial Cell Adhesion Density (Cells/cm²)
Vertical Dimension (nm)
(F)
Glass (Reference)